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Archives of Biochemistry and Biophysics 435 (2005) 182–189
ABB
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Molecular mechanism of the inhibition of cytochrome *c* aggregation by Phe-Gly

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Received 5 November 2004, and in revised form 7 December 2004
Available online 22 December 2004

Abstract

Experimental and computational studies suggest that few general principles govern protein/protein interactions and aggregation. The knowledge of these rules may be exploited to design peptides that are able to interfere with the self-assembly and aggregation of proteins. This work is aimed to verify the validity of this hypothesis by investigating the interaction of cytochrome *c* with Phe and Gly amino acids, Ala-His (carnosine), and two water-soluble dipeptides Phe-Gly and Gly-Phe. The combined use of ^{1}H NMR, MD, and DSC has shown that: (i) at neutral pH, only Phe-Gly is able to prevent the thermally induced aggregation of cytochrome *c*; (ii) Phe-Gly interacts with Gly45 and Phe46 residues of the protein, either when the protein is in the folded or in the unfolded state; and (iii) the interaction of Phe-Gly with cytochrome *c* is sequence-dependent. These results support the hypothesis that the basic principles that describe protein aggregation can be used for the design of peptides with antiaggregating properties.

Keywords: Small chaperone molecules; Aggregation; Folding; Cytochrome *c*

In the crowded environment of the cell, proteins fold spontaneously by avoiding competitive off-pathway processes that lead to aggregation [1,2]. To prevent aberrant protein aggregation, which is associated with a large number of diseases [1,3], nature may employ specific molecular chaperones that actively assist the in vivo folding of proteins. The past decade has seen numerous theoretical and experimental advances in our understanding of how a monomeric protein folds in vitro [4–8]. However, from the perspective of aggregation, it is crucial to understand how proteins may avoid aggregation at physiological or pathological conditions. The early steps of protein aggregation can be described by the formation of protein/protein interfaces which are promoted by favourable interactions between hydrophobic residues. Followingly, the presence of a large number of dangling hydrogen bonds promote intermolecular association. In view of the additional fact that contiguous patches of hydrophobic residues are extremely unlikely to occur in globular proteins [9], it is foreseeable that protein sequences in which hydrophobic residues alternate with amino acids characterized by low steric hindrance are the best candidates to form a nucleus triggering the series of events leading to aggregation. This seeded growth of protein aggregates, which closely resembles the templated assembly envisioned by Griffith [10] to explain the self-replication of infectious prion proteinshas already been verified in simple lattice models [11,12]. To assess the general validity of this hypothesis, in the present paper we investigate the ability of Phe, Gly amino acids, Ala-His (carnosine), Phe-Gly, and Gly-Phe, to specifically bind hydrophobic, poorly structured, solvent accessible regions of cytochrome *c*

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0003-9861/$ - see front matter © 2004 Published by Elsevier Inc.
doi:10.1016/j.abb.2004.12.006

and to inhibit its thermally induced aggregation at neutral pH. To this aim, we screened the potential of the selected peptides to inhibit the thermally induced aggregation of cytochrome *c* by performing DSC measurements according to the following thermal path: heating from 25 to 100 °C, cooling to 25 °C, and reheating to 100 °C. The calorimetric curves showed that only Phe-Gly was able to inhibit cytochrome *c* aggregation and allowed the almost complete refolding of the protein upon cooling. To explain the singular behaviour of Phe-Gly, a set of ^{1}H NMR experiments coupled with molecular dynamics simulations were carried out on the protein/peptide complex. The whole of the results obtained here provides hints supposedly useful either for a better interpretation of the protein aggregation mechanism or for a more finely tuned design of biocompatible, antiaggregating molecules.

Materials and methods

Chemicals

Horse heart cytochrome *c* (purity > 95%), NaH_2PO_4, and Na_2HPO_4 were purchased from Sigma Chemical. Phosphate buffer solutions were prepared daily at pH 7.01, 10 mM ionic strength in 0.1 M NaCl using sterile bidistilled water. Protein solutions were dialyzed overnight against the buffer with a 1000 Da membrane cut-off. Protein concentration was routinely determined by dry weight of the lyophilized protein powder and checked spectrophotometrically using an extinction coefficient $\varepsilon_{410} = 1.06 \times 10^{-5}\,M^{-1}\,cm^{-1}$ at pH 7 and $T = 20\,°C$ [6]. Phe, Gly, Phe-Gly, and Gly-Phe (purity > 95%) were purchased from Bachem (Bubendorf, Switzerland). All inorganic compounds were of analytical grade.

^{1}H NMR spectroscopy

NMR spectra were carried out by a Varian Unity INOVA 500 spectrometer at 499.88 MHz using a tuneable 5 mm Varian inverse probe. Protein and peptide solutions were prepared in D_2O. The protein concentration was 1.2 mg/ml (100 μM) in all experiments. Chemical shifts were referred to the residue signal of HOD as internal reference. All the experiments were carried out at $T = 25\,°C$.

Computer-aided molecular modelling

The native structure of cytochrome *c* was obtained from the Brookhaven Protein Databank (PDB code: 1AKK). Protons were added taking into account the ionization equilibria of the residues at neutral pH at which each simulation was performed. Native cytochrome *c* was then "immersed" in a water sphere consisting of eight layers corresponding to 2000 molecules of solvent and neutralized by adding Na^+ and Cl^- counterions. The final number of atoms was 7964. This model represents a system with a density of about 1.0 g/cm^3. All simulations were carried out in NVT constant conditions. Calculations were carried out by using the CHARMm22 forcefield of the DISCOVER3 module in an INSIGHT II software environment (Accelrys) on an SGI OCTANE R12000 workstation. The system was then optimized by an energy minimization step, using the Polak-Ribiere algorithm, aimed at the elimination of highly strained structures with overlapping atoms. After minimization, a 200 ps molecular dynamics simulation was performed to equilibrate the system using the Velocity Verlet algorithm [13]. Nonbonding interactions were considered by applying an atom-based method with a cut-off of 10 Å for van der Waals interactions and 14 Å for electrostatic forces. The integration timestep was 1 fs for all calculations. The temperature was maintained constant at $T = 298\,K$ by using the Nose–Hoover method [14,15] for each simulation. After equilibration, 1 ns of productive MD simulations were carried out.

To test if the protein/peptide interactions occur in the unfolded state too, we studied a 54-residue peptide that corresponds to residues 1–55 of cytochrome *c* thus encompassing the region in which the peptide/protein interaction is supposed to occur. The random-coil structure adopted by this peptide in isolation reasonably exists in the denatured state of the full-length protein and represents a simplified but realiable model for the investigation of the protein/peptide interaction in the unfolded state. The procedure for docking the dipeptide to native and unfolded cytochrome *c* involved three steps: (i) formation of an initial peptide:protein complex in agreement with NMR data; (ii) 200 ps of equilibration of the obtained complex; and (iii) 1 ns of productive MD simulations of the equilibrated system. The choice of the initial position of the Phe-Gly dipeptide bound to cytochrome *c* focused only on the interactions of residues 45, 46, and 25. In the first step, the peptide was positioned near the selected binding region of cytochrome *c* manually using interactive graphics supported by a computer-generated potential energy grid, which allowed us to rule out high energy geometries. In the second step (equilibration phase), the ligand–host assembly was first surrounded by eight layers of water and then optimized through a minimization step. Followingly, the assembly was equilibrated by 200 ps of MD simulations applying the "rattle" algorithm [16]. The interaction energy between the dipeptide and protein was calculated for each position and the structure with the lowest interaction energy was selected as the new initial configuration for a subsequent equilibration. This iterative process was repeated until the energy difference between the two configurations was less than 20 kcal mol^{-1}. The optimized